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List of Filed Documents

Name of Document Scope of Claims 1

Name of Document Specification 1

Name of Document Drawing 1

Name of Document Abstract 1

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Name of Document Scope of Claims

Claim 1 A method of multiple microorganism detection which isamethodfordetectingtwoormoremicroorganismshavingdifferent properties in foods, with high sensitivity comparable or even superior to official methods, by amplifying a plurality of target genes with a single PCR reaction tube and analyzing the same, comprising the following steps:

(A) a step to culture microorganisms under a culture condition where 1 CFU/100 g microorganisms become 10^3 CFU/ml or more after 24 h of culture;

(B) a step for extracting DNA of the target microorganisms to be detected, by treating at least with a lytic enzyme, a surfactant and a protein denaturant; and

(C) a step for performing Multiplex PCR by mixing a primer specific to the target microorganisms to be detected.

Claim 2 The method of multiple microorganism detection according to claim 1, wherein the two or more microorganisms with different properties comprise Listeria monocytogenes.

Claim 3 The method of multiple microorganism detection according to claim 2, wherein the specific primer is a primer consisting of base sequences shown by SEQ ID Nos: 5 and 6.

Claim 4 The method of multiple microorganism detection according to claim 1, wherein the two or more microorganisms with different properties comprise pathogenic Escherichia coli O157.

Claim 5 The method of multiple microorganism detection according to claim 4, wherein the specific primer is a primer consisting of base sequences shown by SEQ ID Nos: 1 and 2.

Claim 6 The method of multiple microorganism detection according to claim 1, wherein the two or more microorganisms with different properties comprise Salmonella spp.

Claim 7 The method of multiple microorganism detection according to claim 6, wherein the specific primer is a primer consisting of base sequences shown by SEQ ID Nos: 3 and 4.

Claim 8 The method of multiple microorganism detection according to any one of claims 1 to 7, wherein the microorganisms are cultured in a culture condition where the pH after culture becomes 5.1 or more.

Claim 9 The method of multiple microorganism detection according to any one of claims 1 to 8, wherein the microorganisms are cultured in a medium with glucose concentration of 0.15% or less, and/or in a medium with concentration of phosphate-buffer solution of 50 mM or more or in a medium with a buffer ability similar as that with concentration of phosphate-buffer solution of 50 mM or more.

Claim 10 The method of multiple microorganism detection according to any one of claims 1 to 9, wherein the extraction is performed after treating with a lytic enzyme, further treating with a surfactant and a protein denaturant, removing insoluble fractions by centrifugation, and by depositing DNA by alcohol precipitation.

Claim 11 The method of multiple microorganism detection according to any one of claims 1 to 10, wherein the lytic enzyme is Achromopeptidase and/or lysozyme.

Claim 12 The method of multiple microorganism detection according to any one of claims 1 to 11, wherein the surfactant is ethylene oxide condensate of sorbitan monolaurate.

Claim 13 The method of multiple microorganism detection according to any one of claims 1 to 12, wherein the protein denaturant is Guanidine isothiocyanate.

Claim 14 The method of multiple microorganism detection according to any one of claims 1 to 13, wherein Multiplex PCR is performed

by combining the specific primer at a concentration of 120 nM or less.

Claim15Themethodofmultiplemicroorganismdetectionaccording to any one of claims 1 to 14, wherein the food is edible meat or processed meat product.

Name of Document Specification

Title of the Invention Method of multiplex microorganism detection

Technical Field to Which the Invention Pertains

0001

The present invention relates to a method for multiple microorganism detection. This method can detect microorganisms including pathogenic E. coli O157, Listeria monocytogenes and Salmonella spp. existing in foods, with high sensitivity comparable or even superior to official methods, comprising the steps of amplifying a plurality of target genes with a single PCR reaction tube and analyzing the same.

Prior Art

0002

Conventionally, methods of multiple microorganism detection using Multiplex PCR are well known. For example the following methods have been reported: a method of multiple detection of bacteria of pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes, targeting vegetables and fruits (for example, see non-patent document 1); a method of multiple detection of pathogenic Escherichia coli O157 and Salmonella spp. in foods (for example, see non-patent document 2); a method of multiple detection of various bacteria of pathogenic Escherichia coli O157, Salmonella spp., Listeria monocytogenes and Campylobacter spp., targeting milk (for example, see non-patent document 3); a method for multiple detection of Salmonella spp. and Listeria monocytogenes in foods (for example, see non-patent document 4); a method for multiple detection of Escherichia coli including pathogenic Escherichia coli O157, in foods (for example, see non-patent document 5); and a method for multiple detection of bacteria of pathogenic Escherichia coli O157, Salmonella spp.

and Listeria monocytogenes, targeting milk (for example, see non-patent document 6). Further, primers for detecting Escherichia coli (for example, see patent document 1) and primers for detecting O-antigen of pathogenic Escherichia coli O157 (for example, see patent document 2) are known as primers for Multiplex PCR.

0003

On the other hand, as for methods for extracting DNAs of microorganisms, the following methods are known: a method using lytic enzyme Achromopeptidase and the like to mycobacterium such as tubercle bacilli (for example, see patent document 3); a method using lytic enzyme Achromopeptidase and the like to Gram negative bacteria and Gram positive bacteria (for example, see patent document 4); a method using proteinase K or Achromopeptidase and the like to Legionella (for example, see patent document 5); and a method using protein denaturant, reducing agent, surfactant, chelator and the like to Escherichia coli and the like (for example, see patent document 6).

Patent document 1: Japanese Laid-Open Patent Application No. 2001-95576

Patent document 2: Japanese Laid-Open Patent Application No. H11-332599

Patent document 3: Japanese Laid-Open Patent Application No. H6-165676

Patent document 4: Published Japanese translation of PCT international publication No. H9-500793

Patent document 5: Japanese Laid-Open Patent Application No. H5-317033

Patent document 6: Japanese Laid-Open Patent Application No. H6-289016

Non-patentdocument1:JapaneseJournalofFoodMicrobiology, Vol. 19, No. 2, 47-55, 2002

Non-patent document 2: Journal of Industrial Microbiology & Biotechnology, 21, 92-98, 1998

Non-patent document 3: Milchwissenschaft, 55(9), 500-503, 2000

Non-patent document 4: Journal of Food Protection, Vol. 64, No. 11, 1744-1750, 2001

Non-patent document 5: Molecular and Cellular Probes, 13, 291-302, 1999

Non-patent document 6: Food Microbiology, 20, 345-350, 2003Disclosure of the Invention

An Object to be Attained

0004

Methods for detecting one type of microorganism by PCR have been already established, while methods for detecting simultaneously plural microorganisms are being considered in the food sector but no methods sufficiently reliable have been established so far. The object of the present invention is to provide a method of multiple microorganism detection that can detect contaminating microorganisms including pathogenic Escherichia coli O157, Listeria monocytogenes and Salmonella spp. existing in foods, with high sensitivity comparable, or even superior to official methods, comprising the steps of amplifying a plurality of target genes with a single PCR reaction tube, and analyzing the same. In other words, it is to provide a method of multiple microorganism detection that can detect contaminating microorganisms including pathogenic Escherichia coli O157, Listeria monocytogenes and Salmonella spp., by using Multiplex PCR, known as a PCR method using plural pairs of primers

in combination, easily and with high sensitivity and good repeatability.

Means to Attain the Object

0005

(Culture)

Harmful pathogenic bacteria are set up to be “negative” in foods (not included in 25 g), and a comparable or even superior accuracy compared to that of official methods is required for the detection. Enrichment culture is essential to detect microorganisms that have been contaminated in minute amounts such as 1 CFU level in 25 g of food. When performing enrichment culture, usually, culture is conducted by using a selective medium for each target bacterium. However, in order to detect multiple microorganisms simultaneously, considerations were made to proliferate simultaneously plural microorganisms in one type of medium, even for enrichment culture. It is preferable to determine culture conditions leading to detection in the shortest possible time (for example, 24 hours or less), and therefore, the selection of medium becomes particularly important. It is relatively easy to proliferate bacteria simultaneously, when the target microorganisms belong to the same family or bacterial species of same genus, or if their growing properties are alike, but it is difficult for microorganisms of diverse types and having different growing properties. For example, when pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes are the targets to be detected, there was a problem that among these three pathogenic bacteria, Listeria which grows at a low temperature, proliferates slower compared to Salmonella spp. and O157.

0006

Therefore, medium having preferable nutrient source for Listeria, with low carbohydrate and high buffer ability were considered so that Listeria, in particular, can proliferate sufficiently even when other bacteria are contaminated. As a result, Listeria proliferated the fastest of the three contaminated bacteria, in medium No. 17 (triptone 5 g, proteose peptone 5 g, sodium chloride 5 g, glucose 0.5 g, disodium phosphate 7 g, monopotassium phosphate 15 g/1 L), compared to Trypto-soya broth or BHI (Brain Heart Infusion), BPW (Buffered peptone water), and Salmonella spp. and O157 proliferated faster than Listeria. This result suggested that medium No. 17 was preferable for detecting simultaneously these 3 types of bacteria.

(DNA)

At the time of PCR reaction, DNAs are extracted, and lytic operation is necessary for DNA extraction. Lysis of Gram positive bacteria is significantly more difficult compared to that of Gram negative bacteria, as the main bacterial cell wall component is thicker and has a higher-density peptide glycan layer. The present technique was difficult as Listeria, a Gram positive bacterium, was to be detected simultaneously in addition to Gram negative bacteria including Salmonella spp. and O157. Further, from the point of view of extraction from foods, as food residues are various, it is difficult to determine one single lysis method. Particularly, in samples as represented by meat of livestock are high protein and high fat, and have individual difference, lysis is not performed at a constant efficiency, and thus, DNA extraction was difficult. Further consideration revealed that in some Listeria, cells were not disrupted (=DNAs could not be extracted) with only lysozyme, depending on the culture medium, such as Trypto-soya broth or Mueller Hinton Broth. It is estimated

that culture conditions would change according to the type or the deterioration level of foods, even by using the same medium. Further, in some cases, as it is necessary to use media with better recovering ability, extraction methods wherein cells can be disrupted under any circumstances were necessary.

0007

Boiling method and alkaline-SDS method are known as general DNA extraction method, while it was confirmed that Listeria could not be extracted with high sensitivity by the boiling method. SDS is known as a surfactant that is easy to use in DNA extraction, while as it is a strong PCR inhibitor, it is necessary to be removed completely after being used for extraction. For obtaining similar effects and results from an unskilled experimenter as from a skilled artisan, substances that affect the reaction even by a contamination at a low concentration, such as SDS, are not preferable, and SDS has been determined to be a dangerous factor that can be the cause of an extraction efficiency difference with that of a skilled artisan. Further, phenol and chloroform are organic solvents dangerous and harmful to human body and the purification level of DNA becomes better when performing treatment thereof. However, it can be easily estimated that there will be technical individual differences in extraction efficiency, and thus a constant sensitivity cannot be assured. Furthermore, as a particular waste liquid treatment is necessary, it can be said that it is not an extraction method suitable for examination in food manufacturing sites. Therefore, it was necessary to develop a method that is easy and safe so that it can be performed in food manufacturing sites, enabling DNA extraction even from high-protein foods as represented by meat of livestock, that can be easily operated by unskilled or skilled

artisans, and that a constant DNA extraction efficiency (i.e. detection sensitivity) can be expected.

0008

It was shown that bacteria could be lysed completely, by removing large food scraps by passing the culture solution through a 5 µm-filter, adding a lytic enzyme solution (mixed solution of Achromopeptidase and lysozyme), treating the mixture at 37°C for 1 h, and adding a mixed solution of surfactant (Tween 20) and protein denaturant (Guanidine isothiocyanate). DNAs were extracted by removing insoluble fractions by centrifugation, and performing alcohol precipitation. Treatments were necessarily performed as the following order: performing Achromopeptidase treatment prior to or simultaneously with lysozyme treatment; then performing Tween 20 treatment followed by Guanidine isothiocyanate treatment, or performing Tween 20 treatment and Guanidine isothiocyanate treatment simultaneously. It was further shown that as Tween 20 has a high viscosity, it was difficult to add it separately, and thus adding it as a mixture was preferable.

0009

Further, even phenol or chloroform treatment is not performed, protein being soluble to a level sufficient to be detected by PCR without problem can be removed by alcohol precipitation or according to the added level of DNA extraction solution (2 µl per 50 µl of PCR reaction solution). As Tween 20 is also used in PCR reaction solution, there is less inhibition compared to SDS, and thus being easy to be used by unskilled experimenters. Moreover, these extraction methods can be applied for extraction of each bacterium alone, independently. Furthermore, the following methods have been tried: (1) Achromopeptidase alone;

(2) lysozyme alone; (3) Achromopeptidase treatment followed by treatment with guanidine isothiocyanate + Tween 20; (4) lysozyme treatment followed by guanidine isothiocyanate + Tween 20; (5) proteinase K, (6) proteinase K treatment followed by treatment with guanidine isothiocyanate + Tween 20; (7) guanidine isothiocyanate + Tween 20 treatment; (8) guanidine isothiocyanate + Tween 20 + heating treatment. However, any of these methods could not extract Listeria with higher sensitivity, compared to any of the above described methods.

(PCR reaction)

Multiplex PCR was applied as a method for detecting simultaneously plural bacteria. For Multiplex PCR method which is a PCR method performed by combining plural pairing primers, pairing primers that do not mutually produce primer dimmer, or wherein their identification bands do not interfere or overlap each other, and having similar melting temperature were selected and used. It has been revealed that difference arises to the ease of reaction or detection limit according to the primer selection or mixing ratio. When performing Multiplex PCR, it is necessary to adjust the primer mixing ratio in order to detect bands of 3 types of bacteria to be used in later determination, with a similar intensity in the electrophoresis image. When the 3 types of bacteria have the same DNA concentration (20 pg), it was adjusted so that the bands of 3 types of bacteria could be detected with a similar intensity. The most preferable mixing ratio of 6 primers was: 120 nM each of Salmonella spp. primers; 100 nM each of Listeria primers; 80 nM each of O157 primers. Moreover, a mixing ratio with lower concentration, i.e. 30 nM each of Salmonella spp. primers, 25 nM each of Listeria primers and 20 nM each of O157 primers. However, as visual detection by electrophoresis was

difficult though possible, the above concentration was revealed to be preferable.

0010

Further, it is known that target genes can be amplified to approximately 10^{-8} M as final production level by PCR reaction. Normally, approximately 200 nM of primer is added in PCR reaction. It was revealed that this primer level is obviously excessive. Particularly, in case of multiplex reaction, it is highly possible that only results of dominant reaction caused by generated products due to the excessive primer (including non-targeted product) are obtained. Thus, the relationship between the control of the final product level with the multiplex reaction was considered by controlling the primer level in PCR reaction. Naturally, as non-amplified products as represented by primer dimmer also consume primers in PCR reaction, it was revealed that although there is a lower limit of the concentration, more multiplex reactions could be achieved successively by determining the primer concentration to the minimum sensitivity of the detecting machine. In other words, by adjusting the lower limit primer concentration to the sensitivity of the detecting machine, detection of more microorganisms can be expected. By considering the detection machine limit for electrophoresis, fluorescent probe method, or capillary electrophoresis method, the reaction was to be conducted at approximately 100 nM, and detection of 3 types of pathogenic bacteria was confirmed. It is estimated that by increasing the detection machine sensitivity, this concentration can be set to a lower level, and thus, more targets can be detected at once. It was also revealed that if a more sensitive method for detecting amplification products is realized, by controlling the final product level in view of the above, simultaneous multiple

detection by more Multiplex PCR can be realized.

0011

According to the present invention, microorganisms existing in foods, including pathogenic Escherichia coli O157, Listeria monocytogenes and Salmonella spp., can be detected easily with high sensitivity comparable or even superior to official methods, by amplifying a plurality of target genes in a single PCR reaction tube and analyzing the same.

0012

In other words, the present invention relates to: (1) a method of multiple microorganism detection which is a method for detecting two or more microorganisms having different properties in foods, with high sensitivity comparable or even superior to official methods, by amplifying a plurality of target genes with a single PCR reaction tube and analyzing the same, comprising the following steps:

(A) a step to culture microorganisms under a culture condition where 1 CFU/100 g microorganisms become 10^3 CFU/ml or more after 24 h of culture;

(B) a step for extracting DNA of the target microorganisms to be detected, by treating at least with a lytic enzyme, a surfactant and a protein denaturant; and

(C) a step for performing Multiplex PCR by mixing a primer specific to the target microorganisms to be detected; (2) the method of multiple microorganism detection according to (1), wherein the two or more microorganisms with different properties comprise Listeria monocytogenes; (3) the method of multiple microorganism detection according to (2), wherein the specific primer is a primer consisting of base sequences shown by SEQ ID Nos: 5 and 6; (4) the method of multiple microorganism detection according

to (1), wherein the two or more microorganisms with different properties comprise pathogenic Escherichia coli O157; (5) the method of multiple microorganism detection according to (4), wherein the specific primer is a primer consisting of base sequences shown by SEQ ID Nos: 1 and 2; (6) the method of multiple microorganism detection according to (1), wherein the two or more microorganisms with different properties comprise Salmonella spp.; (7) the method of multiple microorganism detection according to (6), wherein the specific primer is a primer consisting of base sequences shown by SEQ ID Nos: 3 and 4.

Moreover, the present invention relates to: (8) the method of multiple microorganism detection according to any one of (1) to (7), wherein the microorganisms are cultured in a culture condition where the pH after culture becomes 5.1 or more; (9) the method of multiple microorganism detection according to any one of (1) to (8), wherein the microorganisms are cultured in a medium with glucose concentration of 0.15% or less, and/or in a medium with concentration of phosphate-buffer solution of 50 mM or more or in a medium with a buffer ability similar as that with concentration of phosphate-buffer solution of 50 mM or more; (10) the method of multiple microorganism detection according to any one of (1) to (9), wherein the extraction is performed after treating with a lytic enzyme, further treating with a surfactant and a protein denaturant, removing insoluble fractions by centrifugation, and by depositing DNA by alcohol precipitation; (11) the method of multiple microorganism detection according to any one of (1) to (10), wherein the lytic enzyme is Achromopeptidase and/or lysozyme; (12) the method of multiple microorganism detection according to any one of (1) to (11), wherein the surfactant is ethyleneoxide condensate of sorbitan

monolaurate; (13) the method of multiple microorganism detection according to any one of (1) to (12), wherein the protein denaturant is Guanidine isothiocyanate; (14) the method of multiple microorganism detection according to any one of (1) to (13), wherein Multiplex PCR is performed by combining the specific primer at concentration of 120 nM or less; (15) the method of multiple microorganism detection according to any one of (1) to (14), wherein the food is edible meat or processed meat product.

Best Mode for Carrying out the Invention

0013

The method of multiple microorganism detection of the present invention is not particularly limited as long as it is a method for detecting two or more microorganisms with different properties in food including edible meat, processed meat products, milk and vegetables, at a high sensitivity comparable or even superior to official methods, by amplifying a plurality of target genes with a single PCR reaction tube and analyzing the same, comprising the following steps: (A) a step to culture microorganisms under a culture condition where 1 CFU/100 g microorganisms become 10^3 CFU/ml or more after 24 h of culture; (B) a step for extracting DNA of the target microorganisms to be detected, by treating at least with a lytic enzyme, a surfactant and a protein denaturant; and (C) a step for performing Multiplex PCR by mixing a primer specific to the target microorganisms to be detected. Further, target microorganisms to be detected are not limited as long as they are contaminating microorganisms in foods, and specifically include, Listeria monocytogenes, pathogenic Escherichia coli O157, Salmonella spp., Campylobacter spp., Vibrio parahaemolyticus, Staphylococcus aureus, Yersinia, Coliform bacteria, Bacillus cereus, Vibrio cholerae, Shigella, Clostridium

botulinum. Further, official methods relate to the methods described in “Guidance for food hygiene inspection” (Japan Food Hygiene Association, 1990), and a specific example is described in Example 9.

0014

According to the multiple microorganism detection method of the present invention, it is possible to detect microorganisms that have been contaminated in minute amounts such as 1 CFU level in 25 g of food, and in the multiple microorganism detection method of the present invention, the culturing process of the contaminating microorganisms in food is indispensable. As for culture conditions of enrichment culture of contaminating microorganisms in food, they are not particularly limited as long as the culture conditions allow 1 CFU/100 g microorganism to become 10^3 CFU/ml or more after 24 h of culture. It is preferable to culture under culture conditions where the pH after culture becomes 5.1 or more using a medium with buffer ability, or to culture in a medium with glucose concentration of 0.15% or less, and/or in a medium with concentration of phosphate-buffer solution of 50 mM or more or in a medium with a buffer ability similar as that with concentration of phosphate-buffer solution of 50 mM or more. As for buffer solution other than phosphate buffer solution, citrate buffer solution, acetate buffer solution, lactate buffer solution, tartrate buffer solution, malate buffer solution, tris buffer solution, MOPS buffer solution and MES buffer solution can be exemplified.

0015

In the multiple detection method of the present invention, the process for extracting DNA of contaminating microorganisms in foods which have been cultured and proliferated is indispensable.

The DNA extraction process is not particularly limited as long as it is a process for extracting DNA of target microorganisms to be detected, by treating at least with a lytic enzyme, a surfactant and a protein denaturant, while a method for depositing and extracting DNA by alcohol precipitation after treating with a lytic enzyme, treating with a surfactant and a protein denaturant, and removing insoluble fractions by centrifugation can be preferably exemplified. Examples of the above lytic enzymes include: Achromopeptidase, lysozyme, proteinase K, chitosanase, chitinase, β-1,3-Glucanase, Zymolyase and Cellulase. One or more of these can be used, while Achromopeptidase and/or lysozyme can be used. As for the above surfactants, anion surfactant, cation surfactant, amphoteric surfactant, nonionic surfactant can be exemplified. Among these, ethylene oxide condensate of sorbitan monolaurate which is a nonionic surfactant, more specifically Tween 20, can be preferably exemplified. As for the above protein denaturants, guanidine isothiocyanate, urea, guanidine hydrochloride, trichloroacetate, SDS, Triton X-100, deoxycholate and the like can be exemplified. One or more of these can be used, while guanidine isothiocyanate is preferable from the view point of bacteriolysis or easy handling. Extract/deposit of DNA from lysates can be performed by commonly known methods such as removing insoluble fractions by centrifugation, and then performing alcohol precipitation.

0016

In the multiple detection method of the present invention, a process comprising the steps of mixing the above extracted DNA and a primer specific to target microorganisms to be detected, to perform Multiplex PCR, is indispensable. As for primers used, it is preferable to select a primer specific to target microorganisms

to be detected, which is a pairing primer having similar melting temperature that do not mutually produce primer dimmer, or wherein their identification bands do not interfere or overlap each other. Further, it is preferable to adjust the mixing ratio of the primers so that bands appearing in electrophoresis used for the subsequent determination can be detected with similar intensity. For example, as for a primer specific for pathogenic Escherichia coli O157, DNA consisting of base sequences shown by SEQ ID NOs: 1 and 2; as for a primer specific for Salmonella spp., DNA consisting of base sequences shown by SEQ ID NOs: 3 and 4; as for a primer specific to Listeria monocytogenes, DNA consisting of base sequences shown by SEQ ID NOs: 5 and 6; are preferable respectively. In this case, it is preferable to mix primers at a total concentration of 600 nM or less, in addition, the most preferable mixing ratio of the 6 primers is: 120 nM each of Salmonella spp. primers, 100 nM each of Listeria primers, 80 nM each of O157 primers.

0017

The detection method after PCR can be performed by, for example, electrophoresis, fluorescent probe method, capillary electrophoresis method, or quantitative PCR method.

In the following, the present invention will be explained more in detail, while the technical scope of the present invention is not limited to these exemplifications.

Example 1

0018

(Simultaneous culture in an existing medium)

Escherichia coli O157: H7 ATCC43894, Salmonella spp. enteritidis IFO3313 and Listeria monocytogenes ATCC49594 were used for pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes, respectively. Further, as for

meat-derived bacteria, 4 strains, Pseudomonas fragi, Citrobacter freundii, Lactobacillus viridescens and Leuconostoc mesenteroides were used. As for a test medium, 2 media, Trypto-soya broth (TSB; Nissui Pharmaceutical) and Buffered Peptone Water (BPW; peptone 10 g, sodium chloride 5 g, sodium hydrogenphosphate 3.5 g, potassium dihydrogenphospate 1.5 g/1 L) were used.

0019

Meat-derived bacteria, and 1 CFU/100 ml each of pathogenic Escherichia coli O157, Salmonella spp., and Listeria monocytogenes were inoculated in the test medium where each bacterium becomes 10^4 CFU/100 ml. After culturing at 35°C, counts of general viable cells, O157, Salmonella spp. and Listeria were counted chronologically. As for general viable cell counts, the total colony counts after culturing at 35°C for 48 h by using a standard agar medium (Nissui Pharmaceutical); as for O157 counts, the Escherichia coli-like colony counts after culturing at 35°C for 24 h by using desoxycholate agar medium (Nissui Pharmaceutical); as for Salmonella spp. counts, the Salmonella spp.-like colony counts after culturing at 35°C for 24 h by using MLCB agar medium (Nissui Pharmaceutical); as for Listeria counts, the Listeria-like colony counts after culturing at 35°C for 48 h by using PALCAM agar medium (Merck) were measured, respectively. The results are shown in Fig. 1. As a result, particularly the proliferation of Listeria monocytogenes was weak in any of the media. By measuring the pH of the culture solution, a decrease of the pH was confirmed.

Example 2

0020

(Influence of buffer ability and sugar concentration of the medium)

As it was thought that the weak proliferation of Lysteria monocytogenes in Example 1 was due to the decrease of the pH

of the medium after culture, the influence of buffer ability and sugar concentration of the medium on the proliferation of each bacterium was investigated. Disodium phosphate and monopotassium phosphate were added to the base medium (triptone 5 g, proteose peptone 5 g, sodium chloride 5 g/1 L) to adjust the phosphate concentration from 15 mM to 200 mM (pH 6.3), and glucose was added by changing the concentration from 0% to 0.25% to prepare the test medium. Meat-derived bacteria and 1 CFU/100 ml each of pathogenic Escherichia coli O157, Salmonella spp. and Listeria moncytegenes, used in Example 1, were inoculated in each test medium where each of these becomes 10^4 CFU/ml. After culturingat35°C,countsofgeneralviablecells,O157,Salmonella spp. and Listeria, and the pH levels were measured-24 h later. The results are shown in Table 1.

0021

As a result, by using a medium with glucose concentration of 0.15% or less, or a medium with phosphate concentration of 50 mM or more, or a medium maintaining the pH after culture at 5.1 or more, all of pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes proliferated to 10^3 CFU/ml or more (cell counts necessary for detection by PCR) by culturing for 24 h. For the tests thereafter, medium No. 17 of Table 1 (triptone 5 g, proteose peptone 5 g, sodium chloride 5 g, glucose 0.5 g, disodium phosphate 7 g, monopotassium phosphate 15 g/1 L) was selected. As for medium components, nitrogen sources other than triptone or proteose peptone, carbon sources other than glucose, substances with buffer ability other than phosphate are also effective according to the existing environment or damage level of the bacteria to be detected. Further, it was more preferable to add inorganic salts, pyruvic acid or pyruvate salt,

orsurfactantssuchasTweenassubstancespromotingproliferation.

0022

Table 1

No.	Phosphate concentration (mM)	Glucose Concentration (%)	General viable cell counts (CFU/ml)	O157 counts (CFU/ml)	Salmonella spp. counts (CFU/ml)	Listeria counts (CFU/ml)	pH	application
1	15	0	2.4×10^{8}	1.4×10^{7}	6.2×10^{6}	2.0×10^{6}	5.99	OK
2		0.5	1.6×10^{8}	1.1×10^{7}	5.6×10^{6}	1.5×10^{6}	5.97	OK
3		1.0	3.0×10^{8}	5.7×10^{6}	4.3×10^{6}	4.7×10^{6}	5.62	OK
4		1.5	2.8×10^{8}	1.4×10^{5}	3.0×10^{5}	3.0×10^{4}	5.11	OK
5		2.0	3.2×10^{8}	5.1×10^{4}	9.1×10^{2}	6.1×10^{2}	4.79	NG
6		2.5	3.9×10^{8}	2.3×10^{4}	1.2×10^{3}	10	4.58	NG
7	30	0	2.1×10^{8}	3.2×10^{7}	5.1×10^{6}	6.2×10^{6}	6.13	OK
8		0.5	1.8×10^{8}	2.7×10^{7}	4.9×10^{6}	5.9×10^{6}	6.10	OK
9		1.0	4.4×10^{8}	2.0×10^{7}	4.8×10^{6}	3.0×10^{6}	5.94	OK
10		1.5	3.1×10^{8}	4.3×10^{6}	7.1×10^{6}	1.6×10^{6}	5.71	OK
11		2.0	6.8×10^{8}	4.8×10^{5}	2.4×10^{4}	6.9×10^{3}	5.40	OK
12		2.5	5.7×10^{8}	4.7×10^{5}	1.6×10^{4}	3.0×10^{2}	5.03	NG
13	50	0.5	1.1×10^{8}	2.4×10^{7}	5.2×10^{6}	6.1×10^{6}	6.12	OK
14		2.5	3.9×10^{8}	1.1×10^{7}	5.0×10^{4}	3.4×10^{4}	5.62	OK
15	100	0.5	3.2×10^{8}	2.5×10^{7}	5.1×10^{5}	5.2×10^{6}	6.10	OK
16		2.5	6.2×10^{8}	2.0×10^{7}	4.2×10^{6}	4.5×10^{6}	6.02	OK
17	150	0.5	4.4×10^{8}	4.1×10^{7}	5.4×10^{6}	5.8×10^{6}	6.17	OK
18		2.5	9.5×10^{7}	3.2×10^{7}	5.1×10^{6}	8.0×10^{6}	6.22	OK
19	200	0.5	1.2×10^{8}	3.2×10^{7}	5.1×10^{6}	5.8×10^{6}	6.17	OK
20		2.5	8.4×10^{7}	3.8×10^{7}	6.8×10^{6}	9.0×10^{6}	6.15	OK

Example 3

0023

(DNA extraction method 1)

Each bacteria of pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes were inoculated to 10 ml of medium No. 17, and cultured at 35°C for 24 h. Each culturesolutionwastakentoa1ml-tube,respectively,centrifuged at 15,000 r.p.m for 5 min to collect bacteria. Lytic enzyme solution {mixed solution of 10 µl of 20 mg/ml Achromopeptidase and 10 µl of 20 mg/ml Lysozyme, and 180 µl of TE buffer [10 mM Tris (hydroxymethyl) aminomethane-hydrochloric acid buffer solution containing 1 mM EDTA, pH 8]} was added to the collected bacteria, treated at 37°C for 1 h. Then, 300 µl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to

2% of Tween 20) was added to dissolve bacteria completely. By observing this solution with a light microscope, it was confirmed that lysis was sufficiently performed. The solution was centrifuged at 15,000 r.p.m for 5 min and 400 μl of the supernatant was transferred to another tube. After precipitating DNA in the lysate with isopropanol, the resultant was centrifuged, and thus, the intended DNA was obtained.

Example 4

0024

(DNA extraction method 2)

Similarly, each bacteria of pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes were inoculated to 10 ml of medium No. 17, and cultured at 35°C for 24 h. Each culturesolutionwastakentoa1ml-tube,respectively,centrifuged at 15,000 r.p.m for 5 min to collect bacteria. 500 μl of lytic agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Tween 20) was added to the collected bacteria to dissolve collected substances. The resultant solution was heated at 100°C for 10 min, and ice cooled for 5 min. By observing this solution with a light microscope, it was confirmed that pathogenic Escherichia coli O157 and Salmonella spp. were lysed, while the lysis level of Listeria monocytogenes was a little lower compared with that of the DNA extraction method of Example 3. Further, lysis of Listeria monocytogenes lysate was performed by respectively using the following: 1) a solution to which a mixed solution of 10 μl of 20 mg/ml Achromopeptidase and 190 μl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 2) a solution to which a mixed solution of 10 μl of 20 mg/ml of Lysozyme and 190 μl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 3) a solution

to which 300 µl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Tween 20) was added to the above 1) solution and mixed; 4) a solution to which 300 µl oflysingagent(4Mguanidineisothiocyanatesolutionsupplemented with 1 to 2% of Tween 20) was added to the above 2) solution and mixed; 5) a solution to which a mixed solution of 1 µl of 20 mg/ml proteinaze K1 and 200 µl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 6) a solution to which 300 µl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Tween 20) was added to the above 5) solution and mixed. The resultants were observed with a light microscope. For all of these, the lysis level of Listeria monocytogenes was a little lower compared with that of DNA extraction method of Example 3.

Example 5

0025

(Determination of PCR reaction condition)

PCR reaction was performed by using the DNA extraction solution obtained in Example 3. For PCR, the following primers were used.

SEQ ID NO: 1: GGC GGA TTA GAC TTC GGC TA

SEQ ID NO: 2: CGT TTT GGC ACT ATT TGC CC

SEQ ID NO: 3: GGG AGT CCA GGT TGA CGG AAA ATT T

SEQ ID NO: 4: GTC ACG GAA GAA GAG AAA TCC GTA CG

SEQ ID NO: 5: CGG AGG TTC CGC AAA AGA TG

SEQ ID NO: 6: CCT CCA GAG TGA TCG ATG TT

The PCR reaction solution was made to be a total of 50 µl by adding 5 µl of 10 × Buffer, 4 µl of dNTP solution, 0.5 µl of UNG, 0.25 µl of AmpliTaq Gold, 10 µl of $MgCl_2$ (all from Applied Biosystems Japan), primers and 2 µl solution of DNA extraction

to sterilized water. The reaction condition is: maintaining for 2 min at 50°C, allowing to react at 95°C for 10 min, repeating 40 times the cycle of 95°C/20 sec, 60°C/30 sec, 72°C/30 sec, maintaining for 7 min at 72°C, and storing at 4°C. PCR products were confirmed by 2.5% agarose gel electrophoresis. The most ideal primer mixing ratio was: 80 nM each of pathogenic Escherichia coli O157 primer (SEQ ID NOs: 1, 2); 120 nM each of Salmonella spp. primer (SEQ ID NOs: 3, 4); 100 nM each of Listeria monocytogenes primer (SEQ ID NOs: 5, 6). For example, a mixing ratio with low concentration, i.e. 20 nM each of pathogenic Escherichia coli O157 primers; 30 nM each of Salmonella spp. primers; 25 nM each of Listeria monocytogenes primers was also investigated, while the visual detection by agarose electrophoresis was somewhat difficult though possible.

Example 6

0026

(Confirmation of reaction specificity)

4 strains of pathogenic Escherichia coli O157, 4 strains of Salmonella spp., 10 strains of Listeria monocytogenes, 4 strains of Escherichia coli other than pathogenic Escherichia coli O157, and 4 strains of Listeria other than Listeria monocytogenes, shown in Table 2 were used to confirm the specificity. Each bacterium was cultured at 35°C for 18 h with Trypto-soya broth (Nissui Pharmaceutical), and the DNA extractions of Example 3 and the PCR reactions of Example 5 were performed. By confirming the result of PCR reactions by 2.5% agarose gel electrophoresis, bands were detected at predetermined places of the electrophoresis image for pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes, and these bacteria were shown to be positive. On the other hand, for Escherichia coli other than pathogenic

Escherichia coli O157, and Listeria other than Listeria monocytogenes, no band was detected and the bacteria were shown to be negative, and it was confirmed that there was no problem to the specificity. The results are shown in Table 2.

0027

Table 2

Bacteria		Results
1-1	Escherichia coli O157:H7	positive
1-2	Escherichia coli O157:H7	positive
1-3	Escherichia coli O157:H7	positive
1-4	Escherichia coli O157:H7	positive
2-1	Salmonella Typhimurium	positive
2-2	Salmonella Enteritidis	positive
2-3	Salmonella Enteritidis	positive
2-4	Salmonella sp.	positive
3-1	Listeria monocytogenes	positive
3-2	Listeria monocytogenes	positive
3-3	Listeria monocytogenes	positive
3-4	Listeria monocytogenes	positive
3-5	Listeria monocytogenes	positive
3-6	Listeria monocytogenes	positive
3-7	Listeria monocytogenes	positive
3-8	Listeria monocytogenes	positive
3-9	Listeria monocytogenes	positive
3-10	Listeria monocytogenes	positive
4-1	Escherichia coli O152	negative
4-2	Escherichia coli	negative
4-3	Escherichia coli	negative
4-4	Escherichia coli	negative
5-1	Listeria welshimeri	negative
5-2	Listeria innocua	negative
5-3	Listeria ivanovii	negative
5-4	Listeria seeligeri	negative

Example 7

0028

(confirmation of detection limit in a meat component-mixed strain)

Pathogenic Escherichia coli O157: H7 ATCC43894, Salmonella spp. IFO3313, and Listeria monocytogenes ATCC49594 were used. Each bacterium was inoculated in 10 ml of the above-mentioned

medium No.17, cultured at 35°C for 24 h, and bacteria culture solution was obtained. Further 225ml of the medium No. 17 was added to 25 g of minced chicken leg, which was ground with a stomacher for 30 sec and cultured at 35°C for 24 h. General viable cell counts in the culture solution at that time were 3.1×10^8 CFU/ml. Culture solution of minced chicken leg was poured by aliquots of 9 ml. Then, 1 ml of 10-fold serial diluted solution of each bacteria culture solution was added to prepare meat sample solution for each diluted series of each bacteria culture solution. After removing large food scraps by passing each sample solution through a 5 μm-filter, the DNA extractions of Example 3 and the PCR reactions of Example 5 were performed, each of them was confirmed by 2.5% agarose gel electrophoresis. ThedetectionlimitofpathogenicEscherichiacoliO157,Salmonella spp. and Listeria monocytogenes in meat sample solution was confirmed to be 10^3 CFU/ml, respectively. The electrophoresis figure showing the result is shown in Fig. 2.

Example 8

0029

(Detection of pathogenic bacteria from inoculated food)

PathogenicEscherichiacoliO157:H7ATCC43894,Salmonella spp. IFO3313, and Listeria monocytogenes ATCC49594 were used. Each bacterium was inoculated so that each of them becomes 10^2 CFU/25 g, 10 CFU/25g, 1 CFU/25 g, 10^{-1} CFU/25 g to minced pork meat. Further 225 ml of the medium No. 17 was added to 25 g of inoculated minced pork meat, which was ground with a stomacher for 30 sec and cultured at 35°C for 24 h. After removing large food scraps by passing each sample solution through a 5 μm-filter, the DNA extractions of Example 3 and the PCR reactions of Example 5 were performed, each of them was confirmed by 2.5% agarose

gel electrophoresis. The result is shown in Fig. 3. As a result, it was confirmed that all of the bacteria could be detected with any of the methods, as long as it exists in an amount of 1 CFU/25 g. Harmful pathogenic bacteria such as pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes, are set up to be “negative” in foods (not included in 25 g), and a comparable or even superior accuracy compared to that of official methods is required for the detection. The present multiple detection method was confirmed to have comparable or even superior accuracy compared to official methods.

Example 9

0030

(Comparable test of official methods with the multiple detection method; detection of pathogenic bacteria from foods on market)

20 samples including chicken meat and chicken liver were purchased at supermarkets. Inspection of pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes were performed by the multiple detection method and the results were compared with those of official methods. Further, the official method was performed as follows.

0031

As for pathogenic Escherichia coli O157, 225 ml of novobiocin mEC broth (Kyokuto Pharmaceutical Industrial) was added to 25 g of sample, which was ground with a stomacher for 30 sec, cultured at 42°C for 18 hours, streaked in CHROMagar O157 medium (Kanto Chemical) and MacConkey Sorbitol Agar medium (Nissui Pharmaceutical), and cultured at 35°C for 18 to 24 h. Those who showed lilac in CHROMagar O157 medium, and translucent pink in MacConkey Sorbitol Agar medium were determined to be false-positive pathogenic Escherichia coli O157, and were streaked

in CLIG agar medium (Kyokuto Pharmaceutical Industrial) and cultured at 35°C for 18 to 24 h. Those whose lower layer was yellow and upper layer was pink, and that were not luminescent to ultraviolet radiation were determined to be false-positive pathogenic Escherichia coli O157. They were subjected to indole reaction, and for those being positive (red), agglutination reaction was performed. Agglutination reaction was performed by using the Escherichia coli O157 detection kit “UNI” (Oxoid). Colonies that were doubtful by agglutination reaction were streaked in CHROMagar O157 medium, MacConkey Sorbitol agar medium, TSI agar medium (Nissui Pharmaceutical) and LIM medium (Nissui Pharmaceutical), and were cultured at 35°C for 24 hours. Those who showed lilac in CHROMagar O157 medium, translucent pink in MacConkey Sorbitol agar medium, yellow in TSI agar medium and showing no change in LIM medium, were confirmed to be pathogenic Escherichia coli O157 by PCR reaction.

0032

As for Salmonella spp., 225 ml of EEM broth (Nissui Pharmaceutical) was added to 25 g of sample, which was ground with a stomacher for 30 sec. The resultant was cultured at 35°C for 18 h, and 1 ml thereof was added to 10 ml of Selenite-Cystine base medium (Nissui Pharmaceutical), and cultured at 43°C for 15 to 18 h. For those wherein the whole or the precipitation showed red, 1-loopful was streaked in MLCB agar medium (Nissui Pharmaceutical) and cultured at 35°C for 24 h. Those who produced black colonies were determined to be false-positive Salmonella spp., and as a confirmation test, these were streaked in TSI agar medium and LIM agar medium. After culturing at 35°C for 24 to 48 h, those who showed red slope, black in upper layer in TSI medium, and showed no change in LIM medium, were determined

to be positive Salmonella spp.

AsforListeriamonocytogenes,225mlofUVMListeriaselective enrichment bouillon (Merck) was added to 25 g of sample, which was ground with a stomacher for 30 sec. After culturing at 30°C for 48 h, 1-loopful was streaked in PALCAM Listeria selective agar medium (Merck). Those who were determined to be positive Listeria after culturing at 35° C for 48 h, were streaked in horse blood agar medium (Nissui Pharmaceutical), Standard agar medium (Nissui Pharmaceutical) and cultured at 35°C for 24 to 48 h. Those whose hemolytic was positive, were subjected to oxydase reaction, catalase reaction, gram staining, microscope observation, Api Listeria (Japan Biomerieux) and those who were identifiedasListeriamonocytogenesweredeterminedtobepositive Listeria monocytogenes.

0033

The multiple detection method was performed as follows. 225 ml of medium No. 17 was added to 25 g of sample, which was ground with a stomacher for 30 sec, and cultured at 35°C for 24 h. After removing large food scraps by passing the culture solution through a 5 μm-filter, the DNA extractions of Example 3 and the PCR reactions of Example 5 were performed, each of them was confirmed by 2.5% agarose gel electrophoresis. The results are shown in Table 3.

0034

As a result, samples in which pathogenic bacteria were detected were as follows. By the official method: pathogenic Escherichia coli O157: 0 sample; Salmonella spp.: 3 samples; Listeria monocytogenes: 6 samples. By any of the multiple detection method: pathogenic Escherichia coli O157: 0 sample; Salmonella spp.: 3 samples; Listeria monocytogenes: 8 samples.

Those who were positive by the official method were all positive by the multiple detection method. Moreover, as for 2 samples of Listeria monocytogenes who were negative by the official method and positive by the multiple detection method (Table 3; sample No. 5, 15), the culture solution in No. 17 was streaked and cultured in PALCAM Listeria selective agar medium (Merck), and the formed colonies were subjected to an identification test. As a result, they were confirmed to be Listeria monocytogenes. From this result, the present multiple detection method was confirmed to have more accuracy than the official method.

0035

Table 3

No.	sample Shop purchased	Name of article	Official method General viable cell count (CFU/g)	O157	Salmonela spp..	Listeria	Multiple detection method O157	Salmonella spp.	Listeria
1	A	Minced red chicken breast (domestic)	$1.7*10^6$	neg	neg	neg	neg	neg	neg
2		Minced poulet breast fillet (domestic)	$3.7*10^7$	neg	neg	neg	neg	neg	neg
3		Minced poulet leg (domestic)	$2.3*10^7$	neg	neg	neg	neg	neg	neg
4		Poulet liver (domestic)	$3.0*10^5$	neg	neg	neg	neg	neg	neg
5	B	Domestic minced poulet fillet	$2.2\text{-}10^5$	neg	pos	neg	neg	pos	pos
6		Domestic chopped poulet let (thawed)	$1.7*10^5$	neg	pos	neg	neg	pos	neg
7		Domestic minced poulet breast	$4.9*10^4$	neg	neg	neg	neg	neg	neg
8		Domestic poulet gizzard	$6.0*10^5$	neg	neg	neg	neg	neg	neg
9	C	Chicken organs (domestic)	$3.6*10^5$	neg	pos	neg	neg	pos	neg
10		Poulet skin (domestic)	$3.4*10^5$	neg	neg	pos	neg	neg	pos
11		Minced poulet (domestic)	$4.8*10^6$	neg	neg	neg	neg	neg	neg
12		Minced poulet fillet (domestic)	$1.6*10^5$	neg	neg	neg	neg	neg	neg
13		Thawed chiken heart (domestic)	$8.6*10^6$	neg	neg	pos	neg	neg	pos
14		Thawed poulet cartilage (thawed	$2.7*10^5$	neg	neg	neg	neg	neg	neg

		US fresh products)							
15	D	Chopped chicken leg (for rice) (domestic)	$1.4*10^6$	neg	neg	neg	neg	neg	pos
16		Poulet leg for fried chicken (S) (product of Brazil)	$6.8*10^7$	neg	neg	pos	neg	neg	pos
17	E	Domestic minced poulet	$3.8*10^6$	neg	neg	neg	neg	neg	neg
18		Domestic muscle-removed poulet filet	$1.2*10^4$	neg	neg	pos	neg	neg	pos
19		Domestic chopped poulet	$2.3*10^5$	neg	neg	pos	neg	neg	pos
20		Domestic poulet sparelib	$2.5*10^6$	neg	neg	pos	neg	neg	pos
			Positive count	0	3	6	0	3	8

Brief Explanation of Drawings

0036

Fig. 1 It is a figure showing change of the counts of pathogenic Escherichia coli O157, Salmonella spp., Listeria monocytogenes and general viable cells in the culture at 35°C using Trypto-soya broth(TSB) and Buffered Peptone Water (BPW).

Fig. 2 It is a figure of the electrophoresis image showing the detection limit by the multiple detection method of each bacterium in the meat sample solution (general viable cell counts: 3.1×10^8 CFU/ml) inoculated with each bacterium of pathogenic Escherichia coli O157, Salmonella spp. and Listeria monocytogenes.

0037

M: molecular weight marker

Lanes 1 to 7 show the pathogenic Escherichia coli-inoculated region (general viable cell counts: 3.1×10^8 CFU/ml), and the inoculated amount of O157 is as follows:

1: 1.1×10^7 CFU/ml; 2: 1.1×10^6 CFU/ml; 3: 1.1×10^5 CFU/ml; 4: 1.1×10^4 CFU/ml; 5: 1.1×10^3 CFU/ml; 6: 1.1×10^2 CFU/ml; 7: 11 CFU/ml

Lanes 8 to 14 show the Salmonella spp.-inoculated region

(general viable cell counts: 3.1×10^8 CFU/ml), and the inoculated amount of Salmonella spp. is as follows:

8: 5.0×10^6 CFU/ml; 9: 5.0×10^5 CFU/ml; 10: 5.0×10^4 CFU/ml;
11: 5.0×10^3 CFU/ml; 12: 5.0×10^2 CFU/ml; 13: 50 CFU/ml;
14: 5 CFU/ml

Lanes 15 to 21 show the Listeria monocytogenes-inoculated region (general viable cell counts: 3.1×10^8 CFU/ml), and the inoculated amount of Listeria monocytogenes is as follows:

15: 1.1×10^7 CFU/ml; 16: 1.1×10^6 CFU/ml;
17: 1.1×10^5 CFU/ml; 18: 1.1×10^4 CFU/ml;
19: 1.1×10^3 CFU/ml; 20: 1.1×10^2 CFU/ml; 21: 11 CFU/ml

Fig. 3 It is a figure of the electrophoresis image showing the result of detection of each bacterium by the multiple detection method, using minced pork meat inoculated with pathogenic EscherichiacoliO157,Salmonellaspp.andListeriamonocytogenes.

0038

M: molecular weight marker

Lanes 1 to 4 show the Listeria monocytogenes-inoculated region, and the inoculated amount of Listeria monocytogenes is as follows:

1: 16 CFU/25g; 2: 1.6 CFU/25g; 3: 0.16CFU/25g;
4: 0.02 CFU/25g

Lanes 5 to 8 show the Salmonella spp.-inoculated region, and the inoculated amount of Salmonella spp. is as follows:

5: 110 CFU/25g; 6: 11 CFU/25g; 7: 1.1 CFU/25g;
8: 0.11 CFU/25g

Lanes 9 to 12 show the pathogenic Escherichia coli O157-inoculated region, and the inoculated amount of O157 is as follows:

9: 85 CFU/25g; 10: 8.5 CFU/25g; 11: 0.85 CFU/25g;

12: 0.09 CFU/25g

Sequence listing

SEQUENCE LISTING

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Name of Document Drawing

Fig. 1

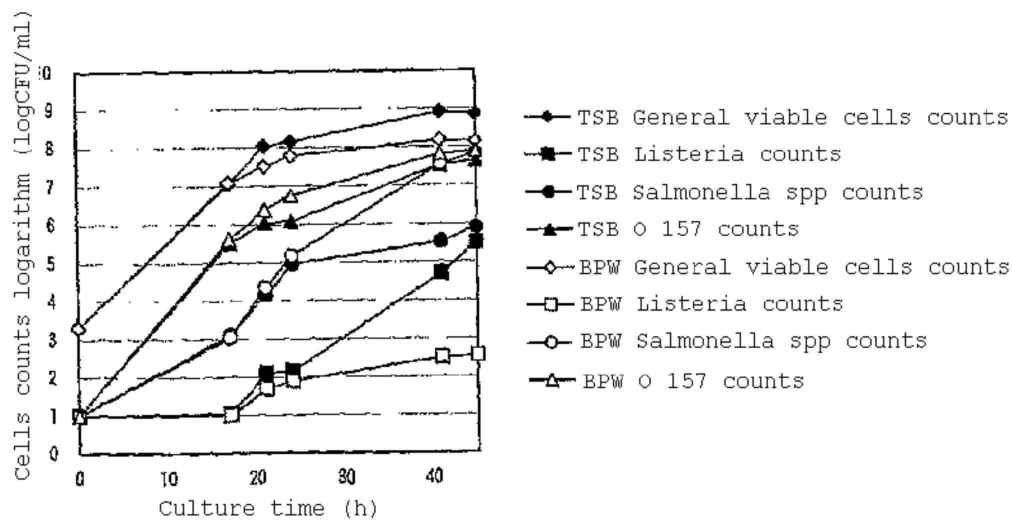


Fig. 2

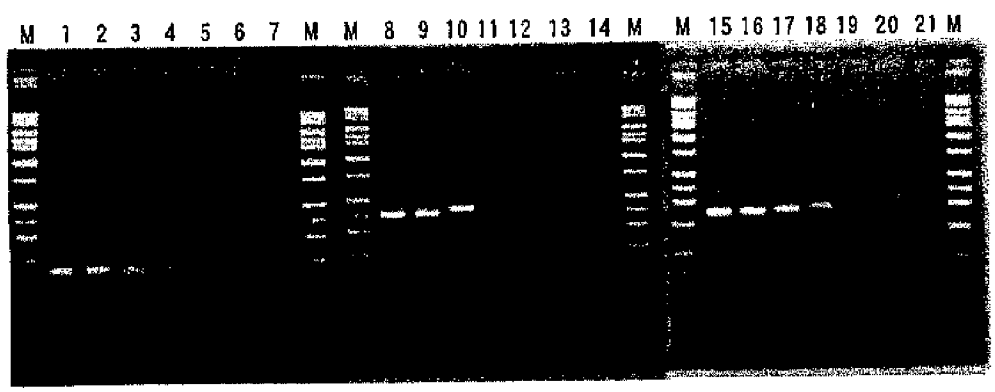
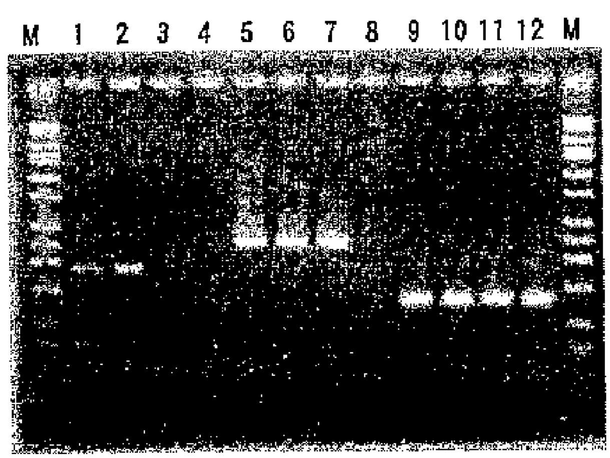


Fig. 3



Name of Document Abstract

Abstract

The Object The object of the present invention is to provide a multiple detection method that can simultaneously detect contaminating microorganisms existing in foods, including pathogenic Escherichia coli O157, Listeria monocytogenes and Salmonella spp., with high sensitivity comparable or even superior to official methods, in a single reaction tube.

Solving Means The following steps are performed: (A) a step to culture microorganisms under a culture condition where 1 to 10 CFU/40ml microorganisms become 10^3 CFU/ml or more after 24 h of culture, for example, under a culture condition where the pH after culture becomes 5.1 or more; (B) a step of extracting DNA of the target microorganisms to be detected by treating with at least a lytic enzyme such as Achromopepidase and/or lysozyme, a surfactant and a protein denaturing agent; and (C) a step of mixing a specific primer to the target microorganisms to be detected to perform Multiplex PCR. Further, two or more microorganisms having different properties in foods are simultaneously detected in a single reaction tube with high sensitivity comparable or even superior to official methods.

DECLARATION

I, Emiko Oku of HIROTA & ASSOCIATES, residing at Wakabayashi Building 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the followings are true and correct translations made by me of the document in the Japanese language filed in Tokyo, Japan for a Japanese patent application under No. 2003-435943 on December 26, 2003 in the name of PRIMA MEAT PACKERS, LTD. and National Agriculture and Food Research Organization entitled: "METHOD OF MULTIPLEX MICROORGANISM DETECTION".

Signed this 22nd day of April, 2010

Emiko Oku

Emiko Oku